REMARKS/ARGUMENTS

Favorable reconsideration and allowance of this application are respectfully requested.

Claims 1-4 and 6-16 are pending in the application with the entry of the foregoing amendments. The claims have been amended in line with the helpful comments and guidance of the examiner in order to recast them in more traditional format and, in some cases, to address the matters set forth in the Office Action. The amended and new claims are supported by the original specification and the attached document of Turner, Microbiology Today, 2000, pages 118-120, which acknowledges that Cyclosporin (Cyclosporin A) is an undecapeptide.

Claims 1-4 and 6 have attracted a Section 112, second paragraph, rejection. As noted above, these claims have been amended to recast them in more traditional format or to address the Section 112 matters in line with the helpful guidance and suggestions of the examiner. The amended claims are believed to moot or obviate the rejection.

Claims 1-3 and 6 stand rejected under 35 U.S.C. §102(b) as allegedly being anticipated by the Wohr article (J. Am. Chem. Soc. 118, 9218, 1996). Although applicants do not necessarily agree with the rejection, they have amended the claims to confirm the patentability of the subject invention and, in many instances, have utilized the helpful suggestions of the examiner as set forth in the Office Action, namely, on pages 6-7 of the Office Action. As noted on page 7 of the Office Action, the claims set forth in the foregoing fashion circumvent the disclosure of Wohr. Applicants submit the same is true with respect to new claims 7-11 (which correspond to the examiner's proposed claim 101) and claims 12-16 (which also relate to the examiner's comments and are further supported by the attached Turner article). As a result, applicants respectfully request the withdrawal of the Section 102 rejection.

In view of the above amendments and remarks, and as supported by the attached document, applicants submit that all pending claims are in condition for allowance and earnestly solicit a notice to that effect. If the examiner has any questions, the undersigned may be contacted at 703-816-4009.

Respectfully submitted,

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Exploitation of fungal secondary metabolites old and new

Geoffrey Turner

Fungal secondary metabolites have been exploited by scientists for many years. Geoffrey Turner describes some current applications and shows how increasing knowledge of fungal gene structure and metabolic pathways is paving the way for the development of new drugs.

The fungal kingdom offers enormous biodiversity, with around 70,000 known species, and an estimated 1·5 million species in total. Most of these are filamentous fungi, which differ from the yeasts not only in their more complex morphology and development (e.g. asexual and sexual structures), but also in their greater metabolic complexity. In particular, they are known for production of secreted enzymes and secondary metabolites, many of which have been exploited by Man. Genetic analysis of secondary metabolic pathways over the past 10 years has revealed some common themes and offered new approaches to the exploitation of natural products.

The best known fungal secondary metabolites in commercial production are the β-lactam antibiotics penicillins G and V and cephalosporin C, produced for over 50 years, with continuous strain and fermentation improvement programmes. During the past 15 years, most of the genes encoding the biosynthetic steps have been characterized, leading to a detailed understanding of the biochemistry and regulation of these pathways. Nevertheless, the long history of traditional strain improvement by mutagenesis and screening had already put into place many of the changes that an applied molecular biologist might have considered after isolating the genes. These include increased gene copy number and enhanced transcription, and limit the scope for further yield improvement. A more sophisticated approach, the engineering of a hybrid cephalosporin pathway in the penicillin producer *Penicillium chrysogenum*, was achieved as an alternative route to semi-synthetic cephalosporins, but its commercial advantage has yet to be established.

Non-ribosomal peptide synthesis

One of the fascinating results of genetic analysis of β-lactam biosynthesis was the discovery that the first step, synthesis of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) from precursor amino acids, was catalysed by a multifunctional enzyme closely related to those responsible for synthesis of the antibiotics gramicidin and tyrocidin by *Bacillus* species. While non-ribosomal peptide biosynthesis and its evolutionary significance had already been described by Fritz Lipmann 'before cloning', gene isolation and DNA sequencing have revealed a large and growing family of peptide synthetases in fungi and bacteria. Although the peptide products show a wide range of biological activity, from antibiotics to pathogenicity factors (Table 1), the biosynthetic mechanism is conserved, and the genes responsible are instantly recognizable from their modular organization (Fig. 1). A module of some 600 amino acids is required for each amino acid incorporated into the peptide. Amino acids are recognized, adenylated, and covalently bound to a module via a 4′-phosphopantetheine cofactor, and less well conserved regions are probably involved in peptide bond formation. The final peptide is released as a linear or cyclic structure, depending on the system.

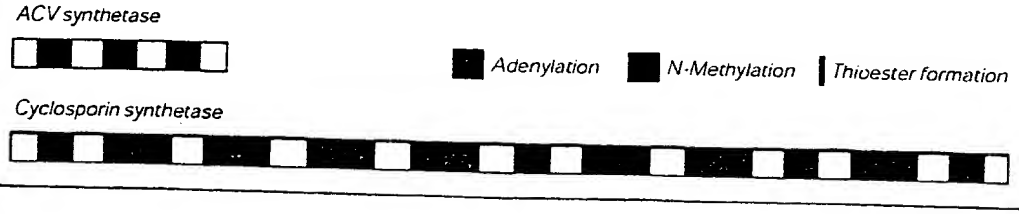
Table 1. Some fungal peptides

Non-ribosomal peptide synthetase genes have been characterized for those shown in bold type.

ACV	***Aspergillus nidulans***
	Penicillium chrysogenum
	Acremonium chrysogenum
Ergotpeptides	***Claviceps purpurea***
Alamethicin	*Trichoderma viride*
Cyclopeptin	*Penicillium cyclopium*
HC-toxin	***Cochliobolus carbonum***
Tentoxin	*Alternaria alternata*
Ferrichrome	*Aspergillus quadricinctus*
Echinocandin	*Aspergillus nidulans*
Cyclosporin	***Tolypocladium inflatum***
Destruxin	***Metarhizium anisopliae***
Enniatin	*Fusarium oxysporum*
Beauvericin	*Beauveria bassiana*

Cyclosporin A, a product of *Tolypocladium inflatum* (Fig. 2), was identified by screening in the 1970s as an antifungal and anti-lymphocytic compound, and exploited as an immunosuppressant, revolutionizing organ transplant surgery. Subsequent studies on its mode of action as an inhibitor of cyclophilin, a peptidyl prolyl isomerase involved in calcium signalling following antigen recognition by T-cells, opened up avenues for discovery of new immunosuppressants. Interestingly, the compound also has anti-*Plasmodium* activity. Cyclosporin is an undecapeptide, assembled by a synthetase consisting of a single polypeptide with a molecular mass of some 1·7 million Da. Some of its 11 modules contain inserted domains responsible for *N*-methylation of the respective

Fig. 1. Modular arrangement in peptide synthetases



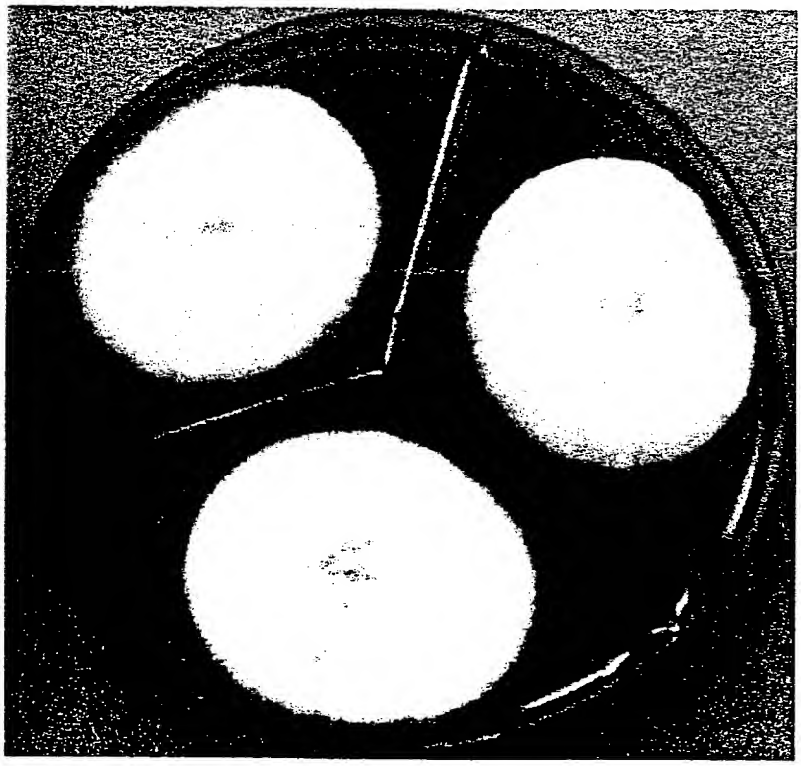
amino acids (Fig. 1). This is one of the largest known enzyme polypeptides, incorporating 40 catalytic functions. While these enzymes appear to use a rather cumbersome way of assembling small peptides, their speciality is their ability to escape the bounds set by ribosomal peptide synthesis. In addition to incorporating valine and alanine, cyclosporin synthetase can *N*-methylate leucine, and incorporate 2-butenyl-4-methyl-L-threonine and α-aminobutyrate.

As medical conditions, including AIDS, resulting in a compromised immune system, have increased in recent years, systemic fungal infections have increased, stimulating a search for better antifungal drugs. The cyclic lipopeptide echinocandin, probably elaborated by a peptide synthetase, is produced by a number of fungi, including a sub-species of *Aspergillus nidulans*. Investigated for some time as an antifungal antibiotic, it interferes with fungal cell wall assembly by inhibiting the synthesis of β-1,3 glucan. Improved semi-synthetic derivatives produced by Eli Lilly and Merck are currently undergoing clinical trials as anti-*Candida* agents.

Ergot alkaloids produced by the plant pathogen *Claviceps purpurea* during infection of rye were responsible for outbreaks of St Antony's Fire, described as long ago as the 9th century. Victims suffered gangrene, convulsions and hallucinations after consuming contaminated rye bread. However, medical applications, such as hastening labour or preventing post-partum bleeding, were recognized from the Middle Ages, and semi-synthetic derivatives such as dihydroergotamine were developed in the 1940s for treatment of blood pressure and migraine. Ergotamine and semi-synthetic derivatives are structural analogues of serotonin and interact with its receptors. Recent studies on the biochemistry and genetics of ergotamine biosynthesis have shown that lysergic acid, synthesized by the fungus, is converted to ergotamine via a three-module peptide synthetase which adds alanine, proline and phenylalanine (Fig. 3).

Polyketide synthetases

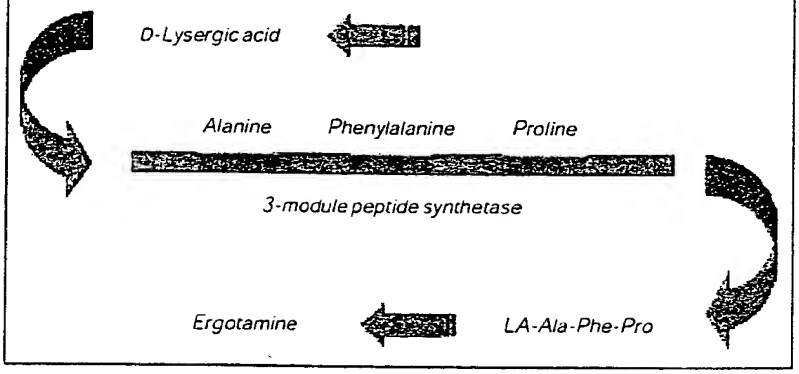
Another major family of multifunctional enzymes responsible for biosynthesis of secondary metabolites are the polyketide synthetases, which are relatives of fatty acid synthetases. While these have been studied most intensively in the prokaryotic actinomycetes, they are also responsible for assembly of potent carcinogens, the aflatoxins of *Aspergillus parasiticus*, and the cholesterol biosynthesis inhibitor lovastatin, produced commercially by *Aspergillus terreus*. Lovastatin is an inhibitor of hydroxymethylglutaryl (HMG) CoA reductase, an early step in cholesterol biosynthesis, and was developed as a treatment for familial hypercholesterolaemia. Subsequently, it has been found to be effective at reducing cholesterol levels in individuals with dietary problems.



LEFT:
Fig. 2. *Tolypocladium inflatum*.
COURTESY BIOCHEMIE GMBH

In the case of both polyketide (PKS) and non-ribosomal peptide synthetases (NRPS), in fungi and bacteria, these enzymes often form only part of a complex pathway involving many other genes for synthesis of precursors, or modification of products. Indeed, there are now some prokaryotic examples of PKS and NRPS modules co-operating to produce secondary metabolites, exemplified by rapamycin, a new immunosuppressant, and yersiniabactin, an iron-chelating siderophore and pathogenicity factor in plague. Elucidation of other pathway components is aided by the common observation that the PKS and NRPS genes are

Fig. 3. **Ergotamine biosynthesis in *Claviceps purpurea***



Further reading

Lipmann, F. (1971). Attempts to map a process evolution of peptide biosynthesis. *Science* 173, 875–884.

Kleinkauf, H. & von Döhren, H. (1996). A nonribosomal system of peptide biosynthesis. *Eur J Biochem* 236, 335–351.

Penalva, M.A., Rowlands, R.T. & Turner, G. (1998). The optimization of penicillin biosynthesis in fungi. *Trends Biotechnol* 16, 483–489.

Tudzynski, P. & others (1999). Evidence for an ergot alkaloid gene cluster in *Claviceps purpurea*. *Mol Gen Genet* 261, 133–141.

Kennedy, J. & others (1999). Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. *Science* 284, 1368–1372.

McDaniel, R. & others (1995). Rational design of aromatic polyketide natural products by recombinant assembly of enzymatic subunits. *Nature* 375, 549–554.

Stachelhaus, T., Schneider, A. & Marahiel, M.A. (1995). Rational design of peptide antibiotics by targeted replacement of bacterial and fungal domains. *Science* 269, 69–72.

located within large gene clusters, which include genes for the other steps. Recent examples include the lovastatin and ergotamine gene clusters.

Future drug discovery

While the examples of useful fungal secondary metabolites described above have been discovered via traditional natural product screening methods, the subsequent genetic analyses suggest alternative approaches based on the highly conserved and easily recognizable module, domain and motif structures of the biosynthetic enzymes. Either genomic data mining or PCR-based approaches could be used to discover new enzymes and secondary metabolic pathways, providing opportunities for product discovery where natural expression is low and the product cannot be easily detected in conventional screens. For example, the Canadian company TerraGen aims to use a gene-based approach to screen fungi which are difficult to culture, such as those found in lichens.

A common feature of commercially exploited fungal secondary metabolites is that natural products have been chemically modified to yield semi-synthetic derivatives. An attractive additional approach would be to redesign the biosynthetic pathways, including the multifunctional enzymes. This approach, using detailed knowledge of gene structure and pathway biochemistry, and taking advantage of the modular structure of the enzymes, has already led to promising progress in the case of polyketide synthetases of the prokaryotic actinomycetes, though success has been more limited so far for peptide synthetases, where a better understanding of enzyme structure and function is needed.

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